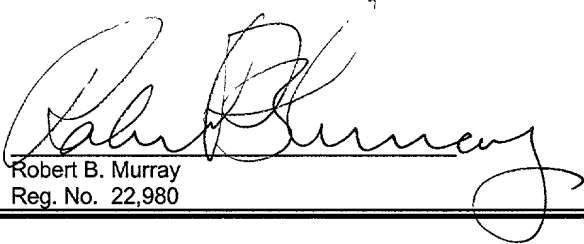


90 Rec'd PCT/PTO 24 OCT 2000

FORM PTO-1390 (REV 5-93)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY DOCKET NO. P101615-00009
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			DATE: October 24, 2000
			U.S. APPLN. NO. (IF KNOWN, SEE 37 CFR 1.5) <b>09/673254</b>
INTERNATIONAL APPLICATION NO. PCT/US99/07016	INTERNATIONAL FILING DATE 22 April 1999	PRIORITY DATE CLAIMED 24 April 1998	
TITLE OF INVENTION: PROCESS FOR PREPARING DOXORUBICIN			
APPLICANT(S) FOR DO/EO/US: Augusto Inventi SOLARI, Giovanna ZANUSO, Silvia FILIPPINI, Francesca TORT, Sharee OTTEN, Anna Luisa COLOMBO, Charles R. HUTCHINSON			
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. (THE BASIC FILING FEE IS ATTACHED)</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT articles 22 and 39(1).</p> <p>4. <input type="checkbox"/> A proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</p> <p>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input type="checkbox"/> have been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input checked="" type="checkbox"/> A copy of the Notification International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11. to 16. below concern other document(s) or information included:</p> <p>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information: PCT/ISA/210, PCT/IPEA/416 CHECK NO. 303534 Drawings - 10 sheets</p>			

U.S. APPLN. NO. (IF KNOWN, SEE 37 C.F.R. 1.50) <b>09/673254</b>		INTERNATIONAL APPLICATION NO. PCT/US99/07016		ATTORNEY DOCKET NO. P101615-00009 DATE: October 24, 2000	
17. <u>XX</u> The following fees are submitted: <b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO.....\$860.00 International preliminary examination fee paid to USPTO (37 CFR 1.492).....\$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.492) but international search fee paid to USPTO (37 CFR 1.492(a)(2)).....\$710.00 Neither international preliminary examination fee (37 CFR 1.492) or international search fee (37 CFR 1.492(a)(3)) paid to USPTO.....\$1,000.00 International preliminary examination fee paid to USPTO (37 CFR 1.492) and all claims satisfied provisions of PCT Article 33(1)-(4) .....\$ 100.00				CALCULATIONS      PTO USE ONLY <hr/>	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$690	
Surcharge of \$130.00 for furnishing the oath or declaration later than _ 20 _ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	19 - 20 =	00	X \$ 18.00	\$00	
Independent Claims	03 - 3 =	00	X \$ 80.00	\$00	
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$00	
TOTAL OF ABOVE CALCULATIONS =				\$690	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$00	
SUBTOTAL =				\$690	
Processing fee of \$130.00 for furnishing the English translation later the _ 20 _ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$00	
TOTAL NATIONAL FEE =				\$690	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$00	
TOTAL FEES ENCLOSED =				\$690	
				Amount to be refunded	\$
				Charged	\$
a. <u>XX</u> A check in the amount of \$690 to cover the above fees is enclosed. b. _ Please charge my Deposit Account No. <u>01-2300</u> in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <u>XX</u> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>01-2300</u> .					
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO:  Arent Fox Kintner Plotkin & Kahn PLLC 1050 Connecticut Avenue, N.W., Suite 600 Washington, D.C. 20036 Telephone No. (202) 857-6000					
				 Robert B. Murray Reg. No. 22,980	

## Process for Preparing Doxorubicin.

### Field of the Invention

The present invention concerns a process for improving daunorubicin to  
5 doxorubicin conversion by means of host cells transformed with recombinant vectors  
comprising DNA encoding a daunorubicin C-14 hydroxylase together with genes  
conferring resistance to anthracycline antibiotics.

### Background of the Invention

Anthracyclines of daunorubicin group such as doxorubicin, carminomycin and  
10 aclacinomycin and their synthetic analogs are among the most widely employed agents  
in antitumoral therapy (F. Arcamone, Doxorubicin, Academic Press New York, 1981,  
pp. 12; A. Grein, Process Biochem., 16:34, 1981; T. Kaneko, Chimicaoggi May 11,  
1988; C. E. Myers et al., "Biochemical mechanism of tumor cell kill" in Anthracycline and  
Anthracedione-Based Anti-cancer Agents (Lown, J. W., ed.) Elsevier Amsterdam,  
15 pp. 527-569, 1988; J. W. Lown, Pharmac. Ther. 60:185, 1993).

Anthracyclines of the daunorubicin group are naturally occurring compounds  
produced by various strains of *Streptomyces* (*S.peucetius*, *S.coeruleorubidus*,  
*S.galilaeus*, *S.griseus*, *S.griseoruber*, *S.insignis*, *S.viridochromogenes*, *S.bifurcus* and  
*S.sp.* strain C5) and by *Actinomyces carminata*. Doxorubicin is mainly produced by  
20 strains of *S. peucetius*. In particular daunorubicin and doxorubicin are synthesized in  
*Streptomyces peucetius* ATCC 29050 and in *S. peucetius subsp. caesius* ATCC 27952.  
The anthracycline doxorubicin is made by *S.peucetius* 27952 from malonic acid,  
propionic acid and glucose by the pathway summarized in Grein, Advan. Applied  
Microbiol. 32:203, 1987 and in Eckart and Wagner, J. Basic Microbiol. 28:137, 1988.  
25 Aklavinone (11-deoxy-e-rhodomyacinone), e-rhodomyacinone, rhodomyacin D,  
carminomycin and daunorubicin are established intermediates in this process. The final  
step in this pathway involves the C-14 hydroxylation of daunorubicin to doxorubicin.

Genes for daunorubicin biosynthesis have been obtained from *S.peucetius*  
29050 and *S.peucetius* 27952 by cloning experiments (Stutzman-Engwall and  
30 Hutchinson, Proc.Natl.Acad.Sci.USA 86:3135,1988; Otten et al., J.Bacteriol.  
172:3427,1990).The gene encoding the daunorubicin 14-hydroxylase, which converts  
daunorubicin to doxorubicin has been obtained from *S.peucetius* 29050 and its mutants  
by cloning experiments and it was overexpressed in the host cells of *Streptomyces*  
species and *Escherichia coli* as described in WO 96/27014, publication date

Sept.6,1996.

Two genes of the daunorubicin biosynthetic cluster, *drrA* and *drrB*, which confer doxorubicin and daunorubicin resistance to *Streptomyces lividans* have been cloned from *S. peucetius* ATCC 29050 strain (Guilfoile and Hutchinson, Proc.Natl.Acad.Sci.USA 88:8553, 1991) (Accession Number M73758 of Genbank) and from the *S.peucetius* 7600 mutant (EP-0371,112-A and Colombo et al., J.Bacteriol.174:1641,1992). These genes encode two translationally coupled proteins, both of which are required for daunorubicin and doxorubicin resistance in this host. The sequence of the predicted product of one of the two genes is similar to the products of other transport and resistance genes, most notably the P-glycoproteins from mammalian tumor cells. Another gene, *drrC*, which confers resistance to daunorubicin and doxorubicin with a strong sequence similarity to the *Escherichia coli* and *Micrococcus luteus* UvrA proteins involved in excision repair of DNA has been cloned from *S.peucetius* ATCC 29050 (Lomovskaya et al., J.Bacteriol.178:3238, 1996).

#### Summary of the invention

The present invention provides a process for improving daunorubicin to doxorubicin conversion in host cells by means of recombinant vectors comprising a DNA region or fragment containing the gene *dxrA* encoding daunorubicin 14-hydroxylase together with a DNA region or fragment containing one, two or three genes, selected from the group consisting of *drrA*, *drrB* and *drrC*, conferring resistance to daunorubicin and doxorubicin. The last three genes confer a high level of resistance in the host cells to doxorubicin, the product of the conversion process, making the process more efficient than the previous one obtained using host cells transformed with the recombinant vectors carrying only the DNA fragment containing the *dxrA* gene, described in WO 96/27014, even when a strong promoter is used.

The DNA of the invention comprises preferably all three of the *drrA*, *drrB* and *drrC* genes or only the two *drrA* and *drrB* genes.

The DNA may be ligated to a heterologous transcriptional control sequence in the correct fashion or cloned into a vector at the restriction site appropriately located

near a transcriptional control sequence in a vector. Typically, the vector is a plasmid. The recombinant vectors may be used to transform a suitable host cell. The host may be strains of Actinomycetes that do not or do produce anthracyclines, preferably strains of *Streptomyces*.

#### 5 Brief description of the drawings

Fig. 1 (a-c) illustrate the construction of the plasmid pIS156 described in Example 1. This plasmid was constructed by insertion of the 2.9 kb fragment containing the *doxA* (formerly *dxrA*), the *dnrV* (formerly *dnrORF10*) and the C-terminal part of the *dnrU* ( $\Delta$ *dnrU*, formerly *dnrORF9*) genes, obtained from the recombinant plasmid pIS70  
10 (WO 96/27014 and A. Inventi Solari et al., GMBIM '96, P58), under the control of the strong promoter *ermE\** (Bibb et al., Molec. Microbiol. 14:533, 1994) into the plasmid pWHM3 (Vara et al., J. Bacteriol. 171:5872, 1989).

In order to better describe the invention, we provide the SEQ.ID. No:1 of 2.867 nt consisting of the *doxA*, *dnrV* and the C-terminal part of the *dnrU* ( $\Delta$ *dnrU*) genes  
15 (complementary strand to the coding strand).

Fig. 2 (a-d) illustrate the construction of the plasmid pIS284 described in Example 1. This plasmid contains the 2.9 kb fragment encompassing the *doxA*, the *dnrV* and the C-terminal part of the *dnrU* genes, obtained from the recombinant plasmid pIS70, under the control of the strong promoter *ermE\** together with a DNA fragment  
20 of 2.3 Kb including the *ddlA* and *ddlB* resistance genes obtained from the plasmid pWHM603 (P. Guilfoile and C.R. Hutchinson, Proc. Natl. Acad. Sci. USA 88:8553, 1991) subcloned into the plasmid pWHM3.

Fig. 3 (a-c) illustrate the construction of the plasmid pIS287 described in Example 2. Said plasmid was constructed by insertion of the 2.9 kb *BamHI-HindIII*  
25 fragment containing the *doxA* formerly, *dxrA*), *dnrV* (formerly *dnr-ORF10*) and the C-terminal part of the *dnrU* ( $\Delta$ *dnrU*, formerly, *dnr-ORF9*) genes, obtained from the recombinant plasmid pIS70 (WO 96/27014), under the control of the strong promoter *ermE\** together with the 2.3 kb *XbaI-HindIII* DNA fragment containing the *ddlA* and *ddlB*

resistance genes and the 3.9 kb *EcoRI-HindIII* fragment containing the *drrC* resistance gene into the plasmid pWHM3.

The maps shown in Figs. 1,2 and 3 do not necessarily provide an exhaustive listing of all restriction sites present in the DNA fragments. However, the reported sites  
5 are sufficient for an unambiguous recognition of the DNA segments.

Restriction sites abbreviations: *Ap*, apramycin; *tsr*, thiostrepton, *amp*, ampicillin; *B*, *BamHI*; *G*, *BglII*; *N*, *NotI*; *K*, *KpnI*; *E*, *EcoRI*; *H*, *HindIII*; *P*, *PstI*; *S*, *SphI*; *X*, *XbaI*; *L*, *BglI*; *T*, *SstI*.

Detailed description of the invention.

10 The present invention provides a DNA molecule in which a DNA region or fragment containing the gene encoding a daunorubicin C-14 hydroxylase is joined to a DNA region or fragment containing one, two or three different genes selected from the group consisting of *drrA*, *drrB*, *drrC* genes encoding proteins conferring to the host cells resistance to daunorubicin and doxorubicin.

15 The DNA region containing the gene encoding a daunorubicin C-14 hydroxylase is preferably the 2.9 kb DNA region obtained from the recombinant plasmid pIS70 described in the patent WO 96/27014 by digestion with *BamHI-HindIII* enzymes. This fragment contains the *doxA* gene, encoding the C-14 hydroxylase. Daunorubicin C-14 hydroxylase converts daunorubicin to doxorubicin. The 2.9 kb DNA fragment also  
20 comprises the *dnrV* gene between the *NotI-KpnI* sites and a *NotI-SphI* fragment containing the C-terminal part of the *dnrU* ( $\Delta$ *dnrU*) gene.

Preferably, this 2.9 kb DNA fragment encoding a daunorubicin C-14 hydroxylase was ligated to both the 2.3 kb *XbaI-HindIII* DNA fragment containing the *drrA* and *drrB* resistance genes obtained from the plasmid pWHM603 and the 3.9 kb *EcoRI-HindIII*  
25 fragment containing the *drrC* gene obtained from the plasmid pWHM264; in another preferred embodiment, the 2.9 kb DNA fragment is ligated to the 2.3 kb *XbaI - HindIII* DNA fragment only.

All the DNA molecules encoding a daunorubicin C-14 hydroxylase described in WO 96/27014 may be employed in the present invention.

In particular the DNA molecule of the present invention may comprise all of the 2.9 kb DNA fragment or only a part of the fragment, at least 1.2 kb in length corresponding to the *KpnI-BamHI* fragment containing the DNA molecule of *doxA*, encoding a daunorubicin C-14 hydroxylase, which converts daunorubicin to doxorubicin.

5 This DNA molecule consists essentially of the sequence reported in the patent application W0 96/27014, which sequence is referred to as the "*dxrA*" sequence. Also, the deduced amino acid sequence of the daunorubicin C-14 hydroxylase is shown in that patent application.

The DNA molecule of the present invention may comprise at least 2247 nt of the 10 2.3 kb *XbaI-HindIII* DNA fragment containing the *drrA* and *drrB* genes encoding proteins conferring to host cells resistance to daunorubicin and doxorubicin.

The DNA molecule of the invention may comprise all or part of the 3.9 kb *EcoRI-HindIII* fragment containing the *drrC* resistance gene, at least 2.5 kb in length corresponding to the *SstI-SphI* fragment containing the DNA molecule of *drrC*, encoding 15 a protein conferring to host cells resistance to daunorubicin and doxorubicin.

The present invention also includes DNA comprising genes conferring resistance to doxorubicin and daunorubicin having a sequence at least 80% identical to the sequences of the *drrA* and *drrB* genes (Guilfoile and Hutchinson, Proc.Natl.Acad.Sci.USA 88:8553, 1991) and or *drrC* gene (Lomovskaya et al., 20 J.Bacteriol.178:3238, 1996).

The DNA molecule of the invention may be ligated to a heterologous transcriptional control sequence in the correct fashion or cloned into a vector at a restriction site appropriately located near a transcriptional control sequence in the vector. Preferably the transcription of the different genes may be coordinated by a 25 common strong promoter such as *ermE\** (Bibb et al., Molec. Microbiol. 14:533, 1994).

The DNA molecule of the invention may be ligated into any autonomously replicating and/or integrating agent comprising a DNA molecule to which one or more additional DNA segments can be added. Typically, however, the vector is a plasmid. A

preferred plasmid is the high-copy number plasmid pWHM3 or pIJ702 (Katz et al., J. Gen. Microbiol. 129:2703, 1983). Other suitable plasmids are pIJ680 (Hopwood et al., Genetic Manipulation of *Streptomyces*. A laboratory Manual, John Innes Foundation, Norwich, UK, 1985) and pWHM601 (Guilfoile and Hutchinson, Proc. Natl. Acad. Sci. USA 88:8553, 1991).

Any suitable technique may be used to insert the DNA into the vector. Insertion can be achieved by ligating the DNA into a linearized vector at an appropriate restriction site. For this, direct combination of sticky or blunt ends, homopolymer tailing, or the use of a linker or adapter molecule may be employed.

The recombinant vector may be used to transform a suitable host cells that do not or do produce anthracyclines.

The host cells may be ones that are daunorubicin or doxorubicin sensitive, i.e., cannot grow in the presence of a certain amount of daunorubicin or doxorubicin, or that are daunorubicin or doxorubicin resistant. In any case the resulting recombinant clones obtained by transformation with the new recombinant vectors of the invention show higher level of resistance to daunorubicin and doxorubicin than the parental host. The level of doxorubicin resistance in recombinant *S. lividans* is much higher than the level observed in anthracycline producing strains *S. peucetius* ATCC 29050 and ATCC 27952.

The host may be a microorganism such as a bacterium. Strains of Actinomycetes, in particular strains of *S. lividans* and other strains of *Streptomyces* species that do not produce anthracyclines may be transformed. *S. lividans* TK 23 is a more suitable host in comparison to the *S. peucetius dnrN* mutant transformed with the recombinant plasmid pIS70 containing the *dxaA* gene used for daunorubicin to doxorubicin bioconversion (WO 96/27014).

The recombinant vectors of the invention may also be used to transform a suitable host cell which produces daunorubicin, in order to enhance the conversion of daunorubicin to doxorubicin.

*S. peucetius* ATCC 29050 and ATCC27952 strains including their mutants that produce



anthracyclines may therefore be transformed. In particular *S. peucetius* strain WMH1654, a mutant strain obtained from *S. peucetius* ATCC 29050 and deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA, under the accession number ATCC55936 may be used.

5 Transformants of *Streptomyces* strains are typically obtained by protoplast transformation.

The invention includes processes for improving doxorubicin production by conversion of daunorubicin, which processes comprise a bioconversion process of added daunorubicin into doxorubicin in hosts which do not produce anthracyclines and  
10 a fermentation process for producing doxorubicin in hosts which directly produce daunorubicin.

Bioconversion process of daunorubicin to doxorubicin.

This process comprises:

- 1) culturing the recombinant host cells not producing daunorubicin transformed with the  
15 vectors of the invention to which daunorubicin is added and
- 2) isolating doxorubicin from the culture.

In this process the recombinant strain may be cultured at temperatures from 20°C to 40°C, for example from 24°C to 37°C. The daunorubicin is added to the culture medium from 24 to 96 hours of the growth phase. The culture is preferably carried out  
20 with shaking. The duration of the culture in the presence of daunorubicin may be from 12 to 72 hours. The concentration of daunorubicin in the culture may be from 20 to 1000 mcg/ml; for example from 100 to 400 mcg/ml.

Doxorubicin production by fermentation.

This process comprises:

- 25 1) culturing recombinant daunorubicin-producing host cells transformed with the vectors of the invention and
- 2) isolating doxorubicin from the culture.

In this process the recombinant strain may be cultured at temperature from 20°C

to 40°C; for example from 26°C to 34°C. The culture is carried out with shaking. The duration of the culture may be from 72 to 168 hours.

### Materials and Methods

- 5 Bacterial strains and plasmids: *E. coli* strain DH5 $\alpha$ , which is sensitive to ampicillin and apramycin is used for subcloning DNA fragments. The host *S. lividans* TK23 was obtained from D. A. Hopwood (John Innes Institute, Norwich, United Kingdom) and the host *S. peucetius* WMH1654 is a mutant strain obtained from *S. peucetius* ATCC 29050 and has been deposited at the American Type Culture Collection, 10801 University  
10 Boulevard, Manassas, Virginia 20110-2209, USA, under the accession number ATCC55936.

The plasmid cloning vectors are pGem-7Zf(+) and related plasmids (Promega, Madison, WI), pIJ4070 (D. A. Hopwood) and the *E. coli-Streptomyces* shuttle vector pWHM3 (Vara et al., J. Bacteriol. 171:5872, 1989).

15

Media and buffer: *E. coli* strain DH5 $\alpha$  is maintained on LB agar (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). When selecting for transformants, ampicillin or apramycin are added at concentrations of 100 micrograms/ml.

- 20 *S. lividans* TK23 and *S. peucetius* WMH1654 are maintained on R2YE (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and ISP4 (Difco, Detroit, MI) agar media, respectively. When selecting for transformants, the plates are overlayed with soft agar containing thiostrepton at a concentration of 50 micrograms/ml.

25

Subcloning DNA fragments: DNA samples are digested with appropriate restriction enzymes and separated on agarose gels by standard methods (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). Agarose slices containing DNA fragments of interest are

excised from a gel and the DNA is isolated from these slices using the GENECLAN device (Bio101, La Jolla, CA) or an equivalent. The isolated DNA fragments are subcloned using standard techniques (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989) into *E. coli* for routine manipulations, and *E. coli-Streptomyces* shuttle vectors or *Streptomyces* vectors for expression experiments.

Transformation of *Streptomyces* species and *E. coli*: Competent cells of *E. coli* are prepared by the calcium chloride method (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989) and transformed by standard techniques (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). *S. lividans* TK23 is grown in liquid R2YE medium (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and harvested after 48 hr. The mycelial pellet is washed twice with 10.3% (wt/vol) sucrose solution and used to prepare protoplasts according to the method outlined in the Hopwood manual (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985). The protoplast pellet is suspended in about 300 microlitres of P buffer (Hopwood et al., *Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985) and 50 microlitres aliquot of this suspension is used for each transformation. Protoplasts are transformed with plasmid DNA according to the small scale transformation method of Hopwood et al. (*Genetic Manipulation of Streptomyces. A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985), Stutzman-Engwall and Hutchinson (Proc. Natl. Acad. Sci. USA. 86:3135, 1988) or Otten et al. (J. Bacteriol. 172: 3427, 1990). After 17 hr of regeneration on R2YE medium at 30°C, the plates are overlayed with 200 micrograms/ml of thiostrepton and allowed to grow at 30°C until sporulated.

Evaluation of daunorubicin and doxorubicin resistance level: The level of resistance is expressed as Minimal Inhibitory Concentration (MIC) and is determined by the standard two-fold dilution method using R2YE medium. The strains are cultured in slants of R2YE medium and incubated at 28°C for 8-10 days. Recombinant strains are grown in the same medium added with 20 micrograms/ml of thiostrepton. Bacterial cultures containing approximately  $10^6$ - $10^7$  viable cells/ml are prepared from cultures grown at 28°C at 280 rpm for 48 hours in Tryptic Soy Broth (Difco). The cultures are homogenized by glass beads. One loopful of the homogenized cultures is inoculated on the agar plates containing different concentrations of daunorubicin and doxorubicin from 0.39 to 800 micrograms/ml. The agar plates are incubated at 30°C for 7 days and the MICs are determined as the lowest concentrations that prevent visible growth.

- Daunorubicin to Doxorubicin bioconversion: *S. lividans* TK23 transformants harboring a plasmid of the invention are inoculated into 25 ml of liquid R2YE medium with 40 micrograms/ml of thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks and incubated on a rotary shaker at 280 rpm at 30 C°. After 2 days of growth, 2.5 ml of this culture are transferred to 25 ml of APM production medium: ((g/l) glucose (60), yeast extract (8), malt extract (20), NaCl (2), 3-(morpholino)propanesulfonic acid (MOPS sodium salt) (15),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.2),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.01),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.01), supplemented with 20 micrograms/ml of thiostrepton. 400 micrograms/ml of daunorubicin are added at 48 hr. of the growth phase. Cultures are grown in 300 ml Erlenmeyer flasks and incubated on a rotary shaker at 280 rpm at 30 C° for 72 hr. Each culture is acidified with 25 milligrams/ml of oxalic acid and after incubation at 30°C on a rotary shaker at 280 rpm for 30 min. is extracted with an equal volume of acetonitrile:methanol (1:1) at 30°C and 300 rpm for 2 hr. The extract is filtered and the filtrate is analyzed by reversed-phase high pressure liquid chromatography (RP-HPLC). RP-HPLC is performed by using a Vydac  $\text{C}_{18}$  column (4.6 x 250 millimeters; 5

micrometers particle size) at a flow rate of 0.385 ml/min. Mobile phase A is 0.2% trifluoroacetic acid (TFA, from Pierce Chemical Co.) in H<sub>2</sub>O and mobile phase B is 0.078% TFA in acetonitrile (from J.T.Baker Chemical Co.). Elution is performed with a linear gradient from 20 to 60% phase B in phase A in 33 minutes and monitored with a diode array detector set at 488 nm (bandwidth 12 micrometers). Daunorubicin and doxorubicin (10 micrograms/ml in methanol) are used as external standards to quantitate the amount of these metabolites isolated from the cultures.

Doxorubicin production: The *S. peuceitius* WMH1654 mutant is transformed with a plasmid of the invention. Transformants are inoculated into 25 ml of R2YE medium supplemented with 20 micrograms/ml thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks on a rotary shaker at 280 rpm at 30°C. After 2 days of growth, 2.5 ml of this culture are transferred to 25 ml of APM medium supplemented with 20 micrograms/ml thiostrepton. Cultures are grown in 300 ml Erlenmeyer flasks on a rotary shaker at 280 rpm at 28°C for 96 - 120 hours. Each culture is acidified with 25 milligrams/ml of oxalic acid and, after 45 min. incubation at 30°C on a rotary shaker at 280 rpm, is extracted with an equal volume of acetonitrile:methanol (1:1) at 30°C and 300 rpm for 2 hr. The extract is filtered and the filtrate is analyzed by RP-HPLC following the same method used to analyze the bioconversion products.

### Example 1

Example 1 (Fig. 1 (a-c) and Fig. 2 (a-d).

In order to remove a non-essential region, the plasmid pIS70 (WO96/27014) is before digested *EcoRI-HindIII* and the 3.5 kb fragment is subcloned into the same sites of the multiple cloning site sequence of the plasmid pGEM-7Zf (+) (Promega, Madison-WI USA) to obtain another *BamHI* restriction site. The new plasmid pGendoxAUV was *BamHI* digested and the fragment, now reduced to 2.9 kb, was transferred into the

plasmid pIJ4070 (from the John Innes Institute, Norwich, UK) under the control of strong promoter *ermE*\*. This new plasmid, named p7doxAUV, was digested *Bgl*III and the fragment inserted into the plasmid pWHM3 (J.Vara et al., J. Bacteriol. 171:5872-5881, 1989) to obtain the plasmid pIS156 (fig. 1c).

- 5 The 2.3 kb *Bgl*III fragment containing the *drrA* and *drrB* resistance genes is transferred after blunt ending from the plasmid pWHM603 into the *Sma*I site of the plasmid pBluescript II SK + (Stratagene) to obtain the plasmid p~~drr~~AB and an *Xba*I-*Hind*III fragment is transferred from p~~drr~~AB into the vector pIJ4070 to obtain pIS278. Afterwards, pIS278 is digested with *Eco*RI-*Xba*I and inserted into the *Eco*RI-*Xba*I
- 10 plasmid pWHM3 to obtain the plasmid pIS281. This plasmid is digested with *Xba*I and the *Xba*I fragment of plasmid pIS156 is inserted to obtain the plasmid pIS284.

### Example 2

- 15 Construction of the plasmid pIS287 (Fig.3 (a-c)): The *drrC* resistance gene contained in the plasmid pWHM264 is excised by *Eco*RI-*Hind*III digestion and inserted into the plasmid pIJ4070 to obtain the plasmid pIS282. From this plasmid, the *drrC* resistance gene is transferred as a *Bgl*III fragment to pIS252 (this plasmid is a modified form of
- 20 pWHM3 containing an extra *Bgl*III site close to the *Eco*RI site) to obtain the plasmid pIS285. pIS285 is *Eco*RI digested and ligated with the 5.5 kb DNA fragment excised from plasmid pIS284 to obtain the plasmid pIS287.

### Example 3

- 25 Resistance of the above recombinant plasmids to doxorubicin: The level of resistance to daunorubicin and doxorubicin of *S. lividans* TK23 transformed with the recombinant plasmids pIS70, pIS284 or pIS287 in comparison with *S. lividans* TK23, *S. lividans* TK23 transformed with the vector pWHM3 and the anthracycline producing *S. peucetius* ATCC 29050 and ATCC 27952 strains is determined as MICs on R2YE

medium following the procedure described in Materials and Methods. The maximum level of daunorubicin and doxorubicin resistance is obtained with the plasmid pIS287 containing the *drrA*, *drrB* and *drrC* resistance genes. The level of doxorubicin resistance was increased 64 times also with the plasmid containing only the *drrA* and *drrB*.resistance genes (Table 1).

**Table 1.** Resistance of recombinant strains to doxorubicin.

Strain	MIC for doxorubicin (micrograms/ml)
<i>S. peucetius</i> ATCC 29050	12.5
<i>S. peucetius</i> ATCC 27952	12.5
<i>S. lividans</i> TK23	12.5
<i>S. lividans</i> TK23(pWHM3)	12.5
<i>S. lividans</i> TK23(pIS284)	800
<i>S. lividans</i> TK23(pIS287)	>800

#### Example 4

Bioconversion of added daunorubicin to doxorubicin in *S. lividans* TK23 transformed with plasmids containing the *doxA* daunorubicin C-14 hydroxylase gene together with different resistance genes: The pIS70, pIS284 or pIS287 plasmids are introduced into *S. lividans* TK23 by transformation with selection for thiostrepton resistance, according to the procedures described in the Materials and Methods section. The resulting *S. lividans* TK23(pIS70), *S. lividans* TK23(pIS284) and *S. lividans* TK23(pIS287) transformants are tested for the ability to bioconvert a high level (400 micrograms/ml) of daunorubicin to doxorubicin using the APM medium as described above. *S. lividans* TK23(pIS70) transformants can convert up to 11.5% of added daunorubicin to doxorubicin (Table 2). *S. lividans* TK23(pIS284) and *S. lividans* TK23(pIS287) transformants can convert up to 73.5% of added daunorubicin to doxorubicin (Table 2).

**Table 2.** Bioconversion of daunorubicin to doxorubicin by *S. lividans* strains.

Strain	Anthracycline (micrograms/ml)		
	DOX	DNR	13-dihydroDNR
5 <i>S. lividans</i> TK23(pIS70) ( <i>control</i> )	46	250	70
<i>S. lividans</i> TK23(pIS284)	294	33	21
<i>S. lividans</i> TK23(pIS287)	288	24	35

**10 Example 5**

Doxorubicin production in the *S. peuceitius* WMH1654 *dnrX* mutant transformed with plasmids containing the *doxA* daunorubicin C-14 hydroxylase gene together with different resistance genes: The pIS284 and pIS287 plasmids are introduced into *S.*

15 *peuceitius* WMH1654 *dnrX* mutant strain by protoplasts transformation with selection for thiostrepton resistance, according to the procedures described in the Materials and Methods section. The resulting *S. peuceitius* transformants are fermented and the fermentation broths analyzed according to the method previously described. *S. peuceitius* WMH1654(pIS284) produced up to 81 micrograms/ml of doxorubicin and up

20 to 18 micrograms/ml of daunorubicin after a 120 hr fermentation (Table 3). *S. peuceitius* WMH1654(pIS287) produced up to 92 micrograms/ml of doxorubicin and no detectable amount of daunorubicin (Table 3).



Table 3. Doxorubicin production by *S. peucetius* WMH1654 *dnrX* strains.

Strain	Anthracycline (micrograms/ml)		
	DOX	DNR	13-dihydroDNR
<i>S. peucetius</i> WMH1654	41	35	18
5 <i>S. peucetius</i> WMH1654(pIS284)	81	18	6
<i>S. peucetius</i> WMH1654(pIS287)	92	0	0

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25 1251 CCCGCCTCCC GGAAGGCGTC GTGCACCTCG GGCTTGCGCT GCATGGTCAT  
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10 2701 AGGTGAGGAT CAGCCGCCCCG CCGCGAGCC GCTCCCGCAG CAGCCGTGCC

15 2751 AGCAGGAAGC CTGCGAGGTG ATTGACCTGG ATGGTGGCCT CGAACCCGTC

2801 CTGGGTCGTG GTGCGCGACC AGAACATGCC GCCGGCGTTG CTGGCCATGA

20 2851 CATCGATGCG CGGGTACCGG

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## CLAIMS

1. A DNA molecule comprising a DNA region containing a gene *doxA* encoding daunorubicin 14-hydroxylase and a DNA region containing at least one gene conferring daunorubicin and doxorubicin resistance.
2. A DNA molecule according to claim 1, further comprising a strong promoter.
3. A DNA molecule according to claim 2, wherein said strong promoter is *ermE\**.
4. A DNA molecule according to claim 1, wherein said gene conferring daunorubicin and doxorubicin resistance is selected from the group consisting of *drrA*, *drrB* and *drrC* genes and any mixtures thereof.
5. A DNA molecule according to claim 4, wherein said genes conferring daunorubicin and doxorubicin resistance are *drrA* and *drrB* genes.
6. The DNA molecule according to claim 4, wherein said genes conferring daunorubicin and doxorubicin resistance are *drrA*, *drrB* and *drrC* genes.
7. The DNA molecule according to claim 1, wherein the region containing the gene *doxA* encoding daunorubicin 14-hydroxylase is 2.9 kb in length.
8. The DNA molecule according to claim 7, wherein the fragment containing the gene *doxA* corresponds to the *KpnI-BamHI* fragment containing the *doxA* nucleotide sequence.
9. The DNA molecule according to claim 5, wherein said region containing said

*drrA* and *drrB* genes is a 2.3 kb *XbaI-HindIII* DNA fragment.

10. The DNA molecule according to claim 1, wherein said genes conferring daunorubicin and doxorubicin resistance are at least 80% identical to genes selected from the group consisting of *drrA*, *drrB* and *drrC* genes.

11. A vector containing a DNA molecule according to claim 1.

12. A vector according to claim 11 wherein said vector is a plasmid.

13. A plasmid according to claim 12, wherein said plasmid is selected from the group consisting of pIS284 and pIS287.

14. A host cell transformed or transfected with a vector according to claim 11.

15. The host cell according to claim 14, wherein said host cell does not produce daunorubicin.

16. The host cell according to claim 14, wherein said host cell is a bacterial cell which produces daunorubicin.

17. The recombinant host cell according to claim 14, wherein said host cell is a *Streptomyces* cell .

18. A process for bioconverting daunorubicin into doxorubicin, comprising the steps of:

culturing a recombinant host cell in a culture medium containing daunorubicin, wherein said host cell contains a DNA molecule comprising a DNA

isolating any resulting doxorubicin from the culture medium.

19. A process for producing doxorubicin by fermentation, comprising the steps of:  
culturing a recombinant host cell in a culture medium, wherein said host cell contains a DNA molecule comprising a DNA region containing a gene *doxA* encoding daunorubicin 14-hydroxylase and a DNA region containing one or more genes conferring daunorubicin and doxorubicin resistance, wherein said host cell is a bacterial cell which produces daunorubicin, and  
isolating any resulting doxorubicin from the culture medium.



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SEQUENCE LISTING

&lt;110&gt; PHARMACIA &amp; UPJOHN S.P.A.

&lt;120&gt; PROCESS FOR PREPARING DOXORUBICIN

&lt;130&gt; 1615-9003

&lt;140&gt; PCT/UNKNOWN

&lt;141&gt; 1999-04-22

&lt;150&gt; 09/065, 606

&lt;151&gt; 1998-04-24

&lt;160&gt; 1

&lt;170&gt; PatentIn Ver. 2.0

&lt;210&gt; 1

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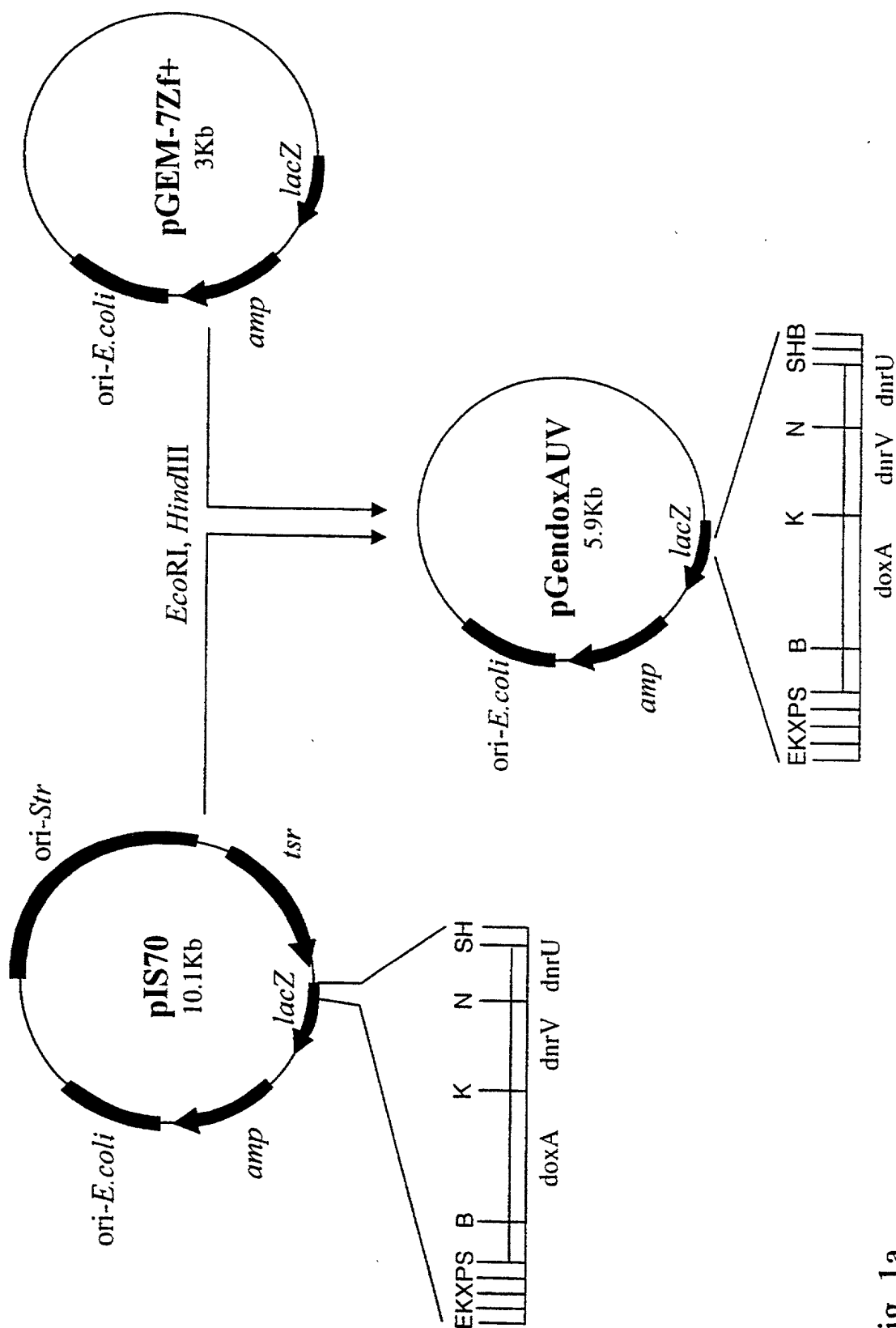


Fig. 1a

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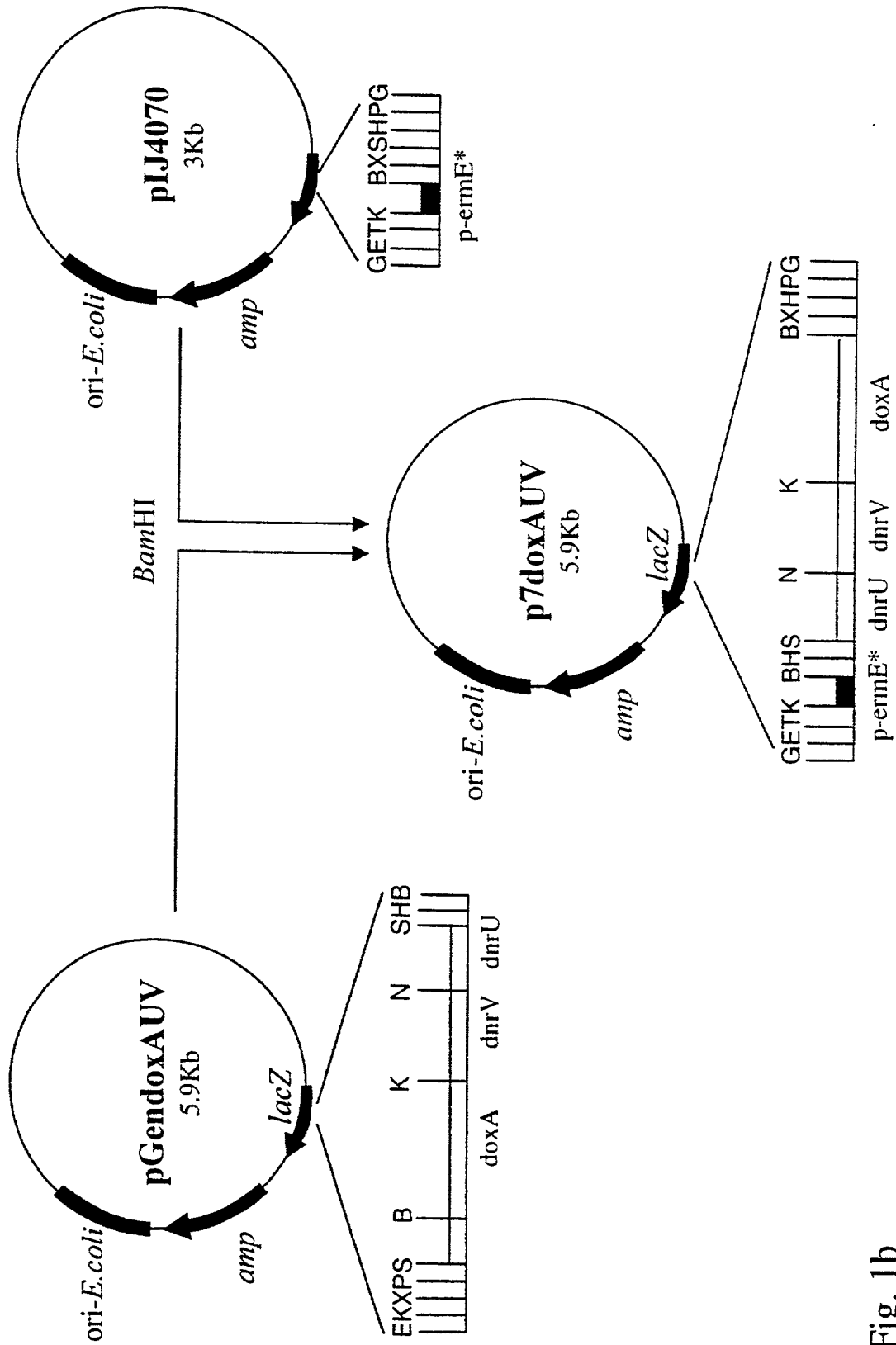


Fig. 1b

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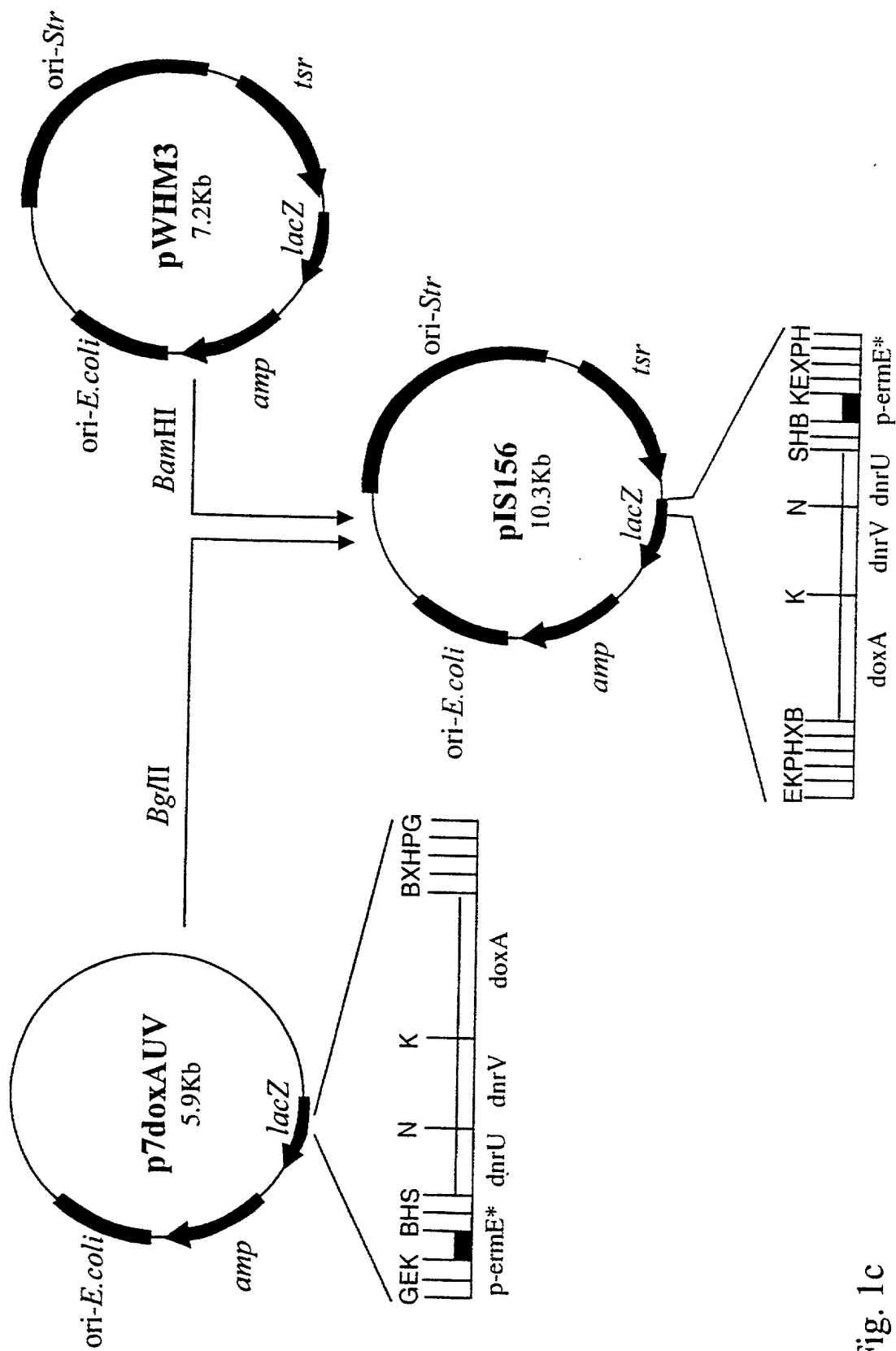


Fig. 1c

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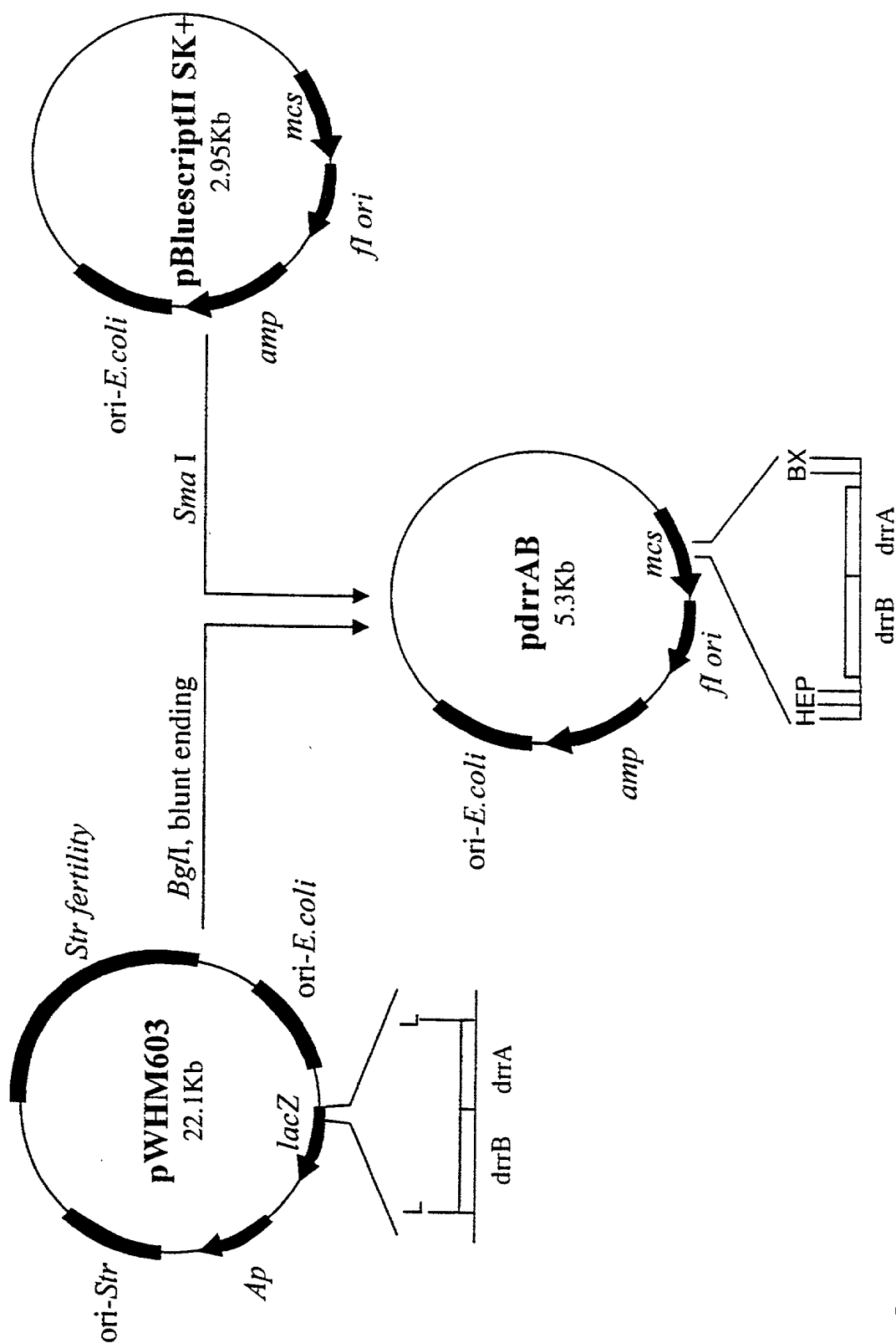


Fig. 2a

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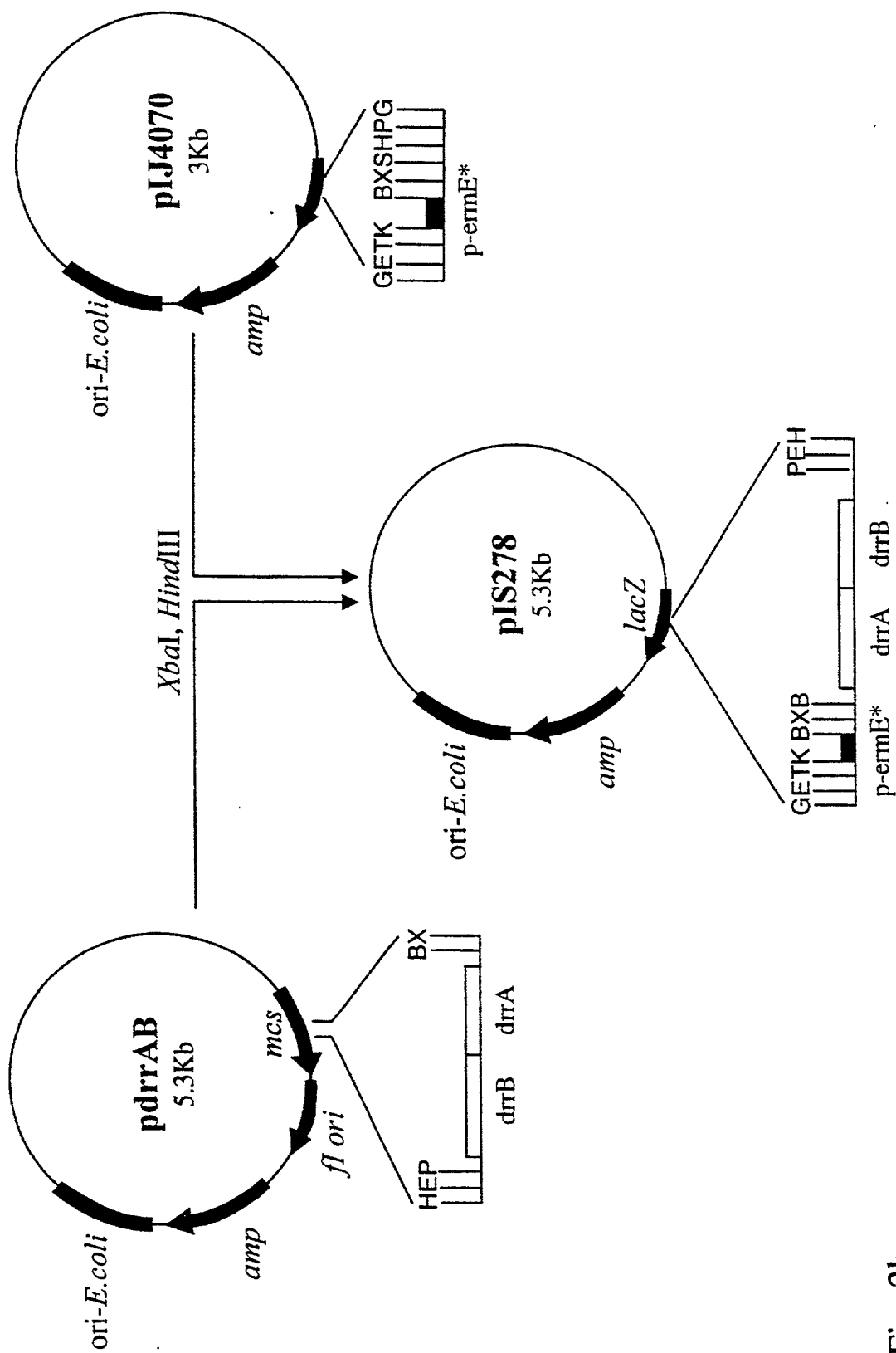


Fig. 2b

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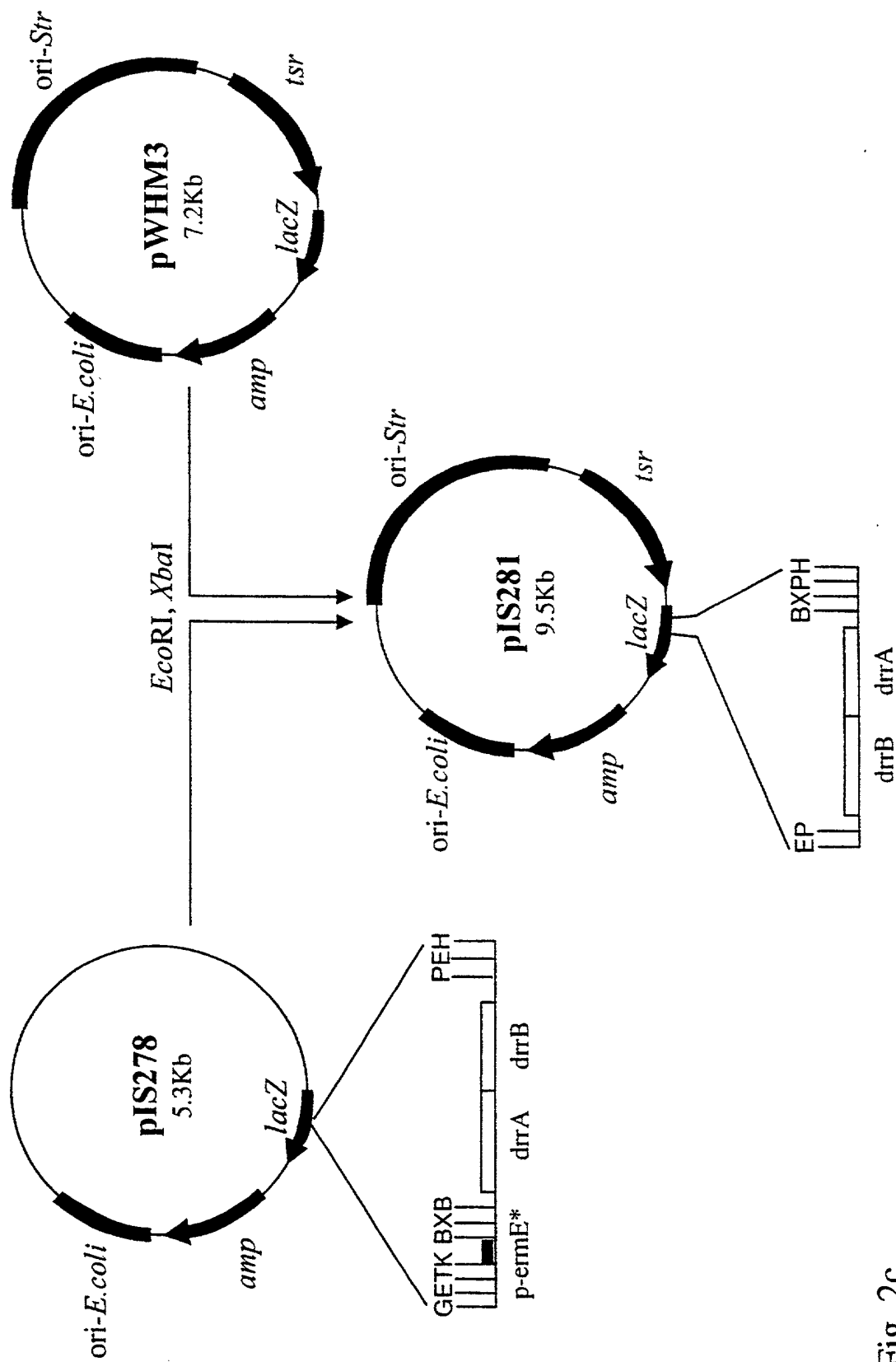


Fig. 2c



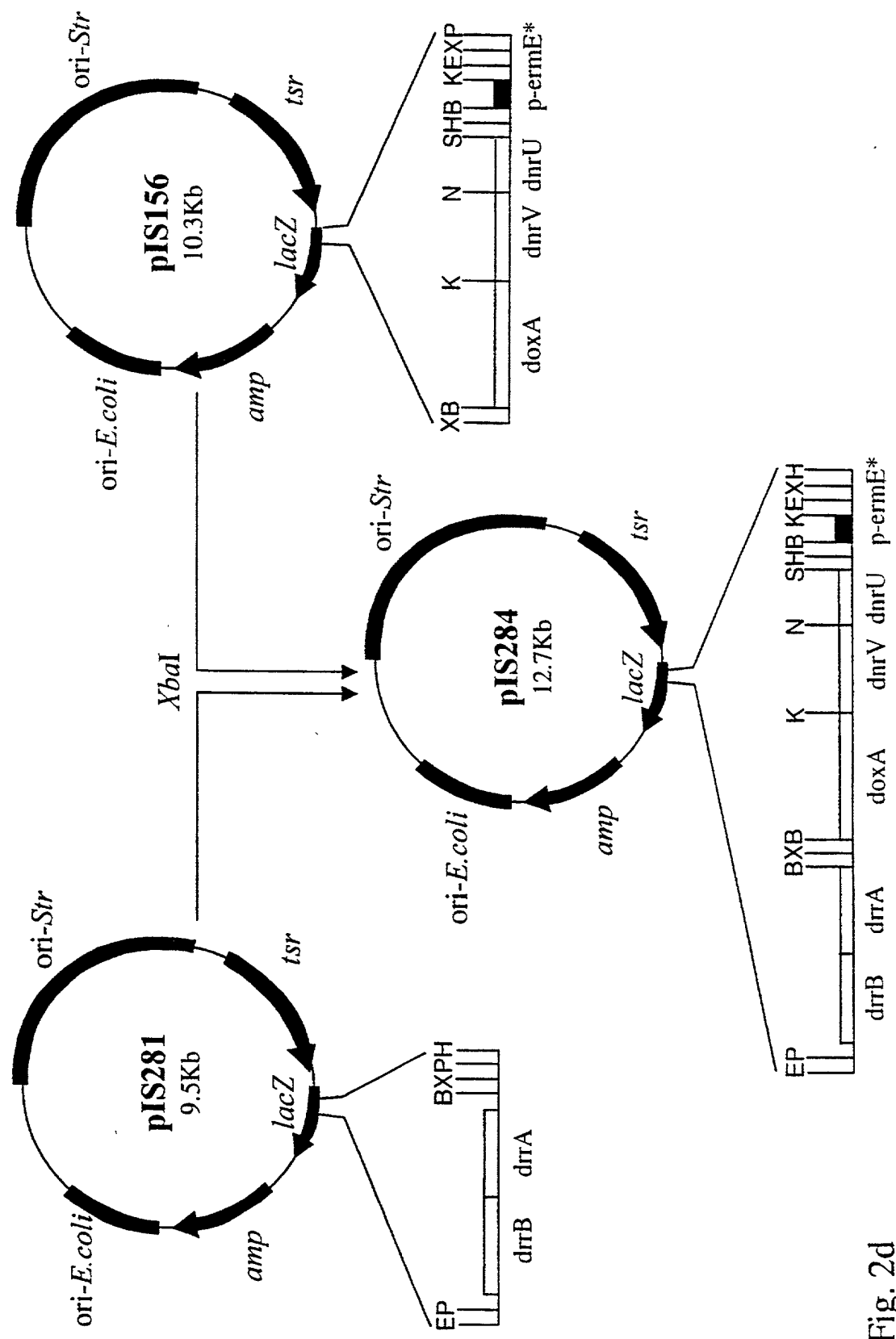


Fig. 2d

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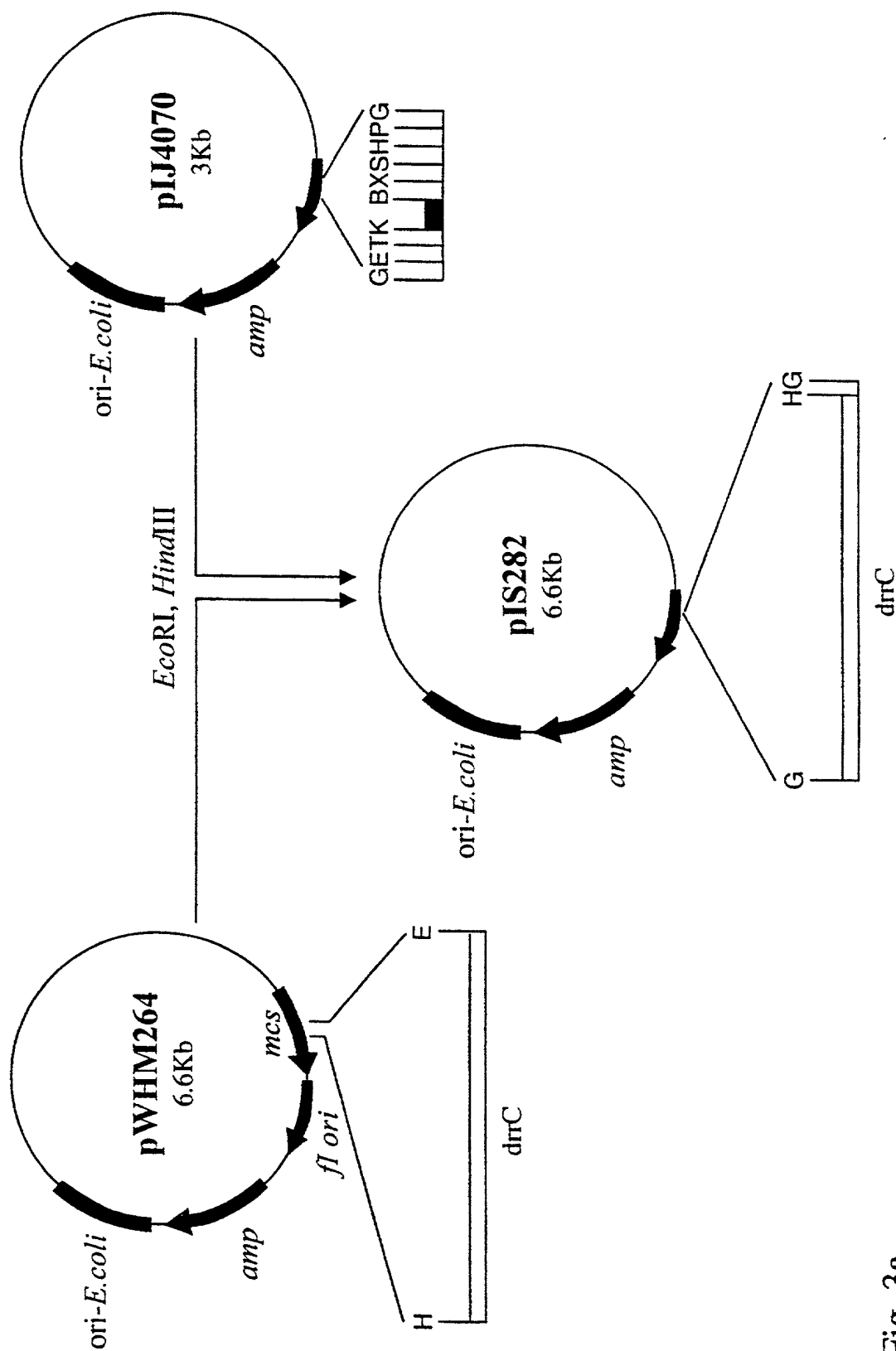


Fig. 3a

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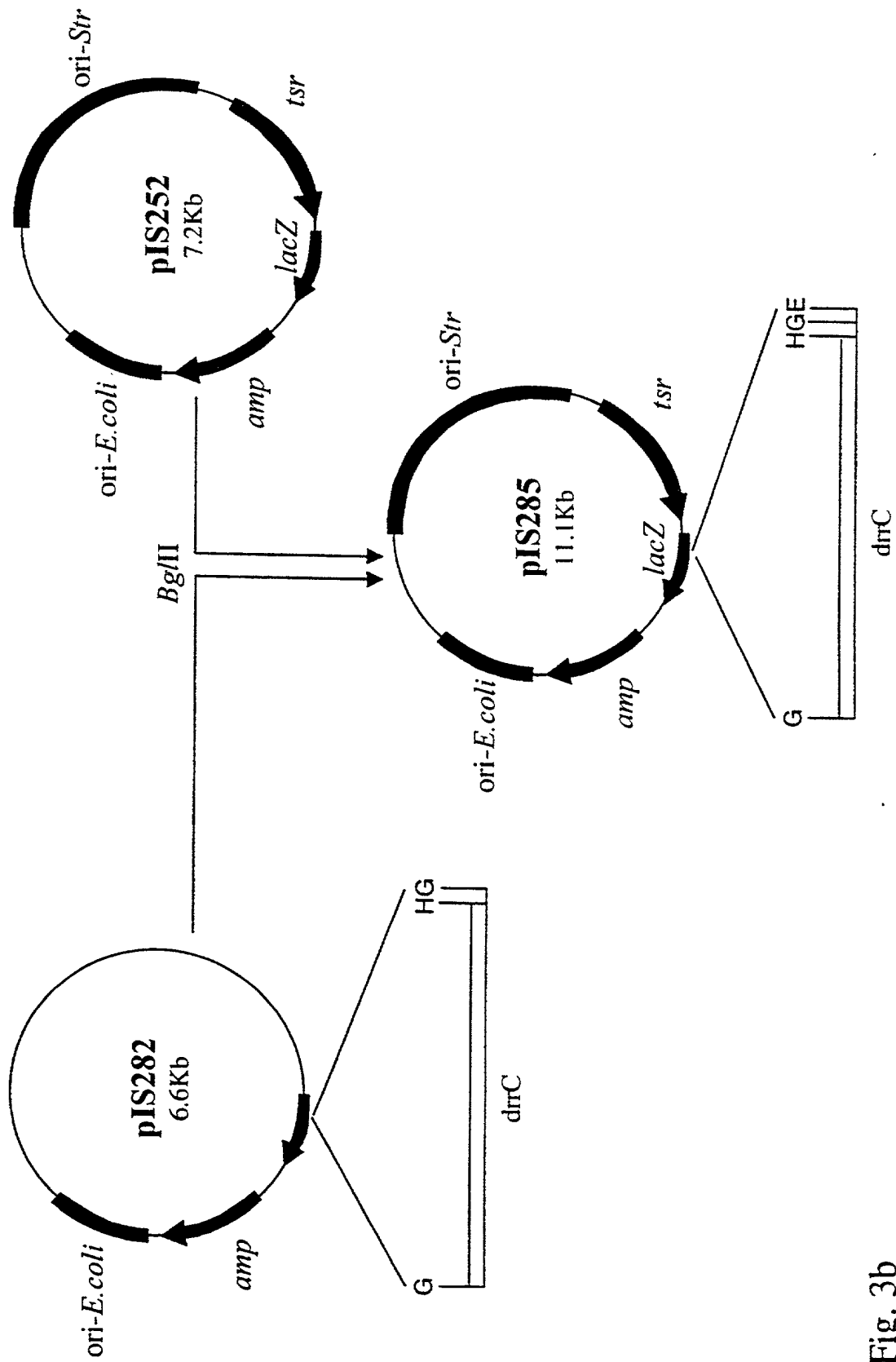


Fig. 3b

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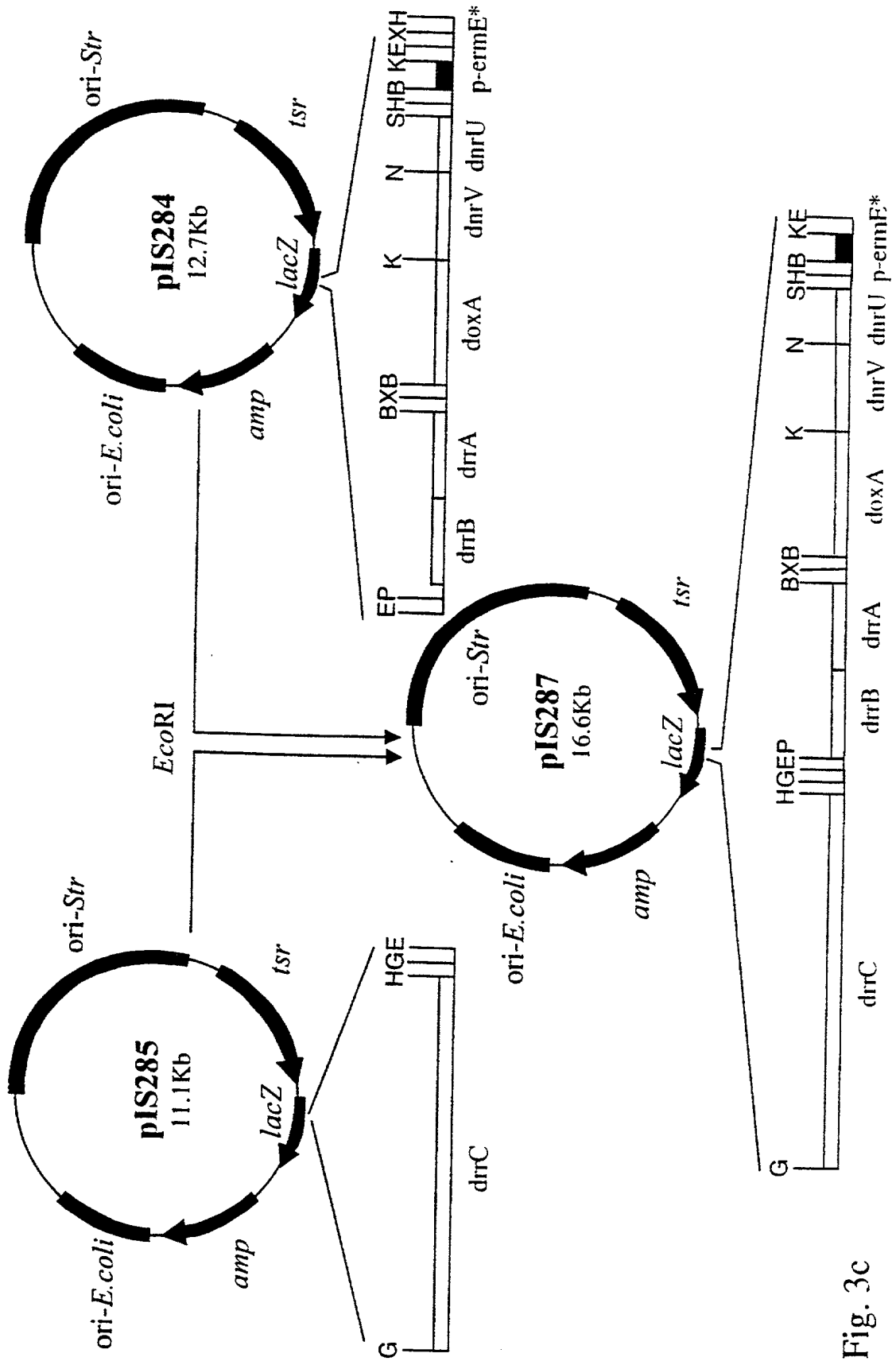


Fig. 3c

## Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

(Insert Title) PROCESS FOR PREPARING DOXORUBICIN

the specification of which is attached hereto unless the following box is checked:

- ☒ was filed on April 22, 1999 as PCT International Application Number PCT/US99/07016 and was amended on

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

(List prior foreign applications. See note A on back of this page)	<u>09/065,606</u> (Number)	<u>US</u> (Country)	<u>24 April 1998</u> (Day/Month/Year Filed)	Priority Claimed <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
	<u></u> (Number)	<u></u> (Country)	<u></u> (Day/Month/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No
	<u></u> (Number)	<u></u> (Country)	<u></u> (Day/Month/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

<u></u> (Application Number)	<u></u> (Filing Date)
<u></u> (Application Number)	<u></u> (Filing Date)

(See Note B on back of this page)

- ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)	<u></u> (Application Serial No.)	<u></u> (Filing Date)	<u></u> (Status) (patented, pending, abandoned)
	<u></u> (Application Serial No.)	<u></u> (Filing Date)	<u></u> (Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys: Robert B. Murray, Reg. No. 22,980; David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Douglas H. Goldhush, Reg. No. 33,125; Monica Chin Kitts, Reg. No. 36,105; Richard J. Berman, Reg. No. 39,107; King L. Wong, Reg. No. 37,500; James A. Poulos, III, Reg. No. 31,714; Murat Ozgu, Reg. No. 44,275; Bradley D. Goldizen, Reg. No. 43,632; N. Alexander Nolte, Reg. No. 45,689; Robert K. Carpenter, Reg. No. 34,794; Gregory B. Kang, Reg. No. 45,273; Rustan I. Hill, Reg. No. 37,351; Rhonda L. Barron, Reg. No. P47,271; Carl Schaukowitz, Reg. No. 29,211; and Kevin F. Turner, Reg. No. 43,437.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

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Date

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Inventor's signature Giovanna Zanuso

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Date

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Inventor's signature Silvia Filippini

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000021-15224960

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SECRET

7W

